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54 **Thermostable human Cu/Zn superoxide dismutase muteins.**

57 **Thermostable muteins of human Cu/Zn superoxide dismutase (hSOD) in which one or both of the free cysteine residues at positions 6 and III are replaced with an uncharged amino acid. hSOD Ala6, hSOD SerIII, and hSOD Ala6 SerIII are specifically exemplified.**

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THERMOSTABLE HUMAN Cu/Zn SUPEROXIDE DISMUTASE MUTEINSDescriptionTechnical Field

This invention is in the fields of protein chemistry and genetic engineering. More particularly it relates to muteins of human Cu/Zn superoxide dismutase in which either or both of the free cysteines at amino acid positions 6 and III of the native protein have been replaced with a neutral amino acid.

Background

Superoxide dismutases are a family of enzymatic proteins that include associated metal ions. The members of the family differ in both amino acid sequence and in the type of associated metals. Their ability to catalyze the destruction of superoxide ions renders them useful as pharmaceuticals, in cosmetics, and in food preservation. The amino acid sequence of human Cu/Zn superoxide dismutase (hereinafter referred to as hSOD) is described in Jabusch et al, *Biochemistry* (1980) 19:2310-2316. The cloning and sequencing of hSOD cDNA and the production of hSOD in bacteria and yeast are described in EPA 84111416.8 (published 24 April 1985 under number 0 138 III).

One potential shortcoming of hSOD is its relative lability to heat. This property limits its shelf life and its ability to be made into pharmaceutical, food, or cosmetic formulations that may be subjected to elevated temperatures. In this regard, it is known that yeast Cu/Zn SOD contains no free cysteines, but is less thermostable than bovine SOD or hSOD (Steinman, H. M. in *Superoxide Dismutase* Vol I, pp 18-19, CRC Press, 1982). The only reference relating to increasing the thermal stability of hSOD is Jabusch et al, supra. It reports that alkylation of Cys III of native hSOD with iodoacetate yields a more stable molecule.

The only reference that describes a mutein of hSOD is Hallewell et al, *Nucleic Acids Research* (1985) Vol 13, No 6, pp 2017-2034. It reports a hSOD mutein having an Ala → Glu substitution at position four. No dismutation activity was detected for that mutein and it was thus estimated that its activity was reduced more than 20-fold relative to the native molecule.

Cysteine modifications have been made in other molecules. For instance, U.S. Pat No. 4,518,584 describes muteins of lymphokines in which cysteines that are not essential for biological activity are replaced with a neutral amino acid to facilitate the production of molecules with proper disulfide bridging in bacteria. Perry and Wetzel, *Science* (1984) 226:555-557 describe a mutein of T4 lysozyme having an Ile → Cys substitution at position three, a disulfide link between positions 3 and 97, and a free cysteine at position 54. Alkylation of that free cysteine increased the thermal stability of the mutein.

In sum the art suggests that alkylation of Cys III of native hSOD increases thermal stability, but is silent as to whether an amino acid substitution at position III or other positions would result in a functional molecule and whether such a change would increase the thermostability of the molecule. Indeed, the only reported mutein of hSOD lacked dismutase activity and the relative thermostabilities of yeast, bovine and human SODs appear to indicate that thermostability is dependent upon something other than the free cysteine content of the molecule.

Disclosure of the Invention

Applicants have found that the substitution of either or both of the free cysteines at positions 6 and III of hSOD with an uncharged (neutral) amino acid enhances the thermal stability of the molecule. hSOD with both of these cysteines replaced is significantly more thermostable than hSOD with only a single cysteine replaced. Hence, hSOD with both cysteines replaced is preferred.

Accordingly, muteins of hSOD in which at least one of the cysteine residues at positions 6 and III is replaced with an uncharged amino acid are one aspect of the invention.

Another aspect of the invention is a method of enhancing the thermostability of hSOD comprising replacing at least one of the cysteine residues at positions 6 and III of hSOD with an uncharged amino acid.

Pharmaceutical and cosmetic compositions containing such muteins are yet another aspect of the invention.

The DNA, expression vectors and recombinant organisms that are used to make the above described muteins are also part of the invention.

Brief Description of the Drawings

Figure 1 shows the sequence of a DNA fragment that encodes hSOD and the amino acid sequence of hSOD.

Figure 2 is a flow chart for the construction of plasmid pYSODA, a yeast expression plasmid used to produce a mutein of hSOD having a Cys → Ala substitution at position 6 of the amino acid sequence shown in Figure 1 (the mutein is designated hSOD Ala6).

Figure 3 is a flow chart for the construction of plasmid pSODCFI, an intermediate used in the construction of plasmid pYSODAS described below.

Figure 4 is a flow chart for the construction of the mutant M13 clone M13rp8SODC11S, an intermediate construct used to prepare plasmid pYSODS described below.

Figure 5 is a flow chart for the construction of plasmid pYSODS, a yeast expression plasmid used to produce a mutein of hSOD having a Cys → Ser substitution at amino acid position III of the sequence shown in Figure 1 (the mutein is designated hSOD SerIII).

Figure 6 is a flow chart for the construction of plasmid pYSODAS, a yeast expression plasmid for producing a mutein of hSOD having a Cys → Ala substitution at position 6 and a Cys → Ser substitution at position III (the mutein is designated hSOD Ala6 SerIII).

Figures 7-9 are photographs of various electrophoretic gel analyses described in the examples.

Figures 10-12 are graphs depicting the relative thermal stabilities of hSOD and the hSOD muteins described in the examples.

Modes for Carrying Out the Invention

The hSOD muteins of the invention have at least one of the two free cysteine residues at positions 6 and III (amino acid numbering herein refers to that of the native molecule shown in Figure 1) replaced with an uncharged (nonpolar or uncharged polar), preferably acyclic (nonaromatic, nonheterocyclic) amino acid. Amino acids having hydrogen (i.e., glycine), an aliphatic, or a hydroxylic side chain are particularly preferred replacements. When both cysteines are replaced, the two replacement amino acids may be the same or different. Examples of amino acids that may be used to replace the cysteines are glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, methionine, proline, tryptophan, and tyrosine. Serine and alanine are preferred replacement residues. Other amino acids of the native hSOD sequence may be replaced or deleted provided that such alterations neither affect adversely the dismutase activity of the mutein nor its thermostability. The number of such alterations will usually be less than about 5.

Specific examples of muteins of the invention are hSOD Ala6, hSOD Gly6, hSOD Val6, hSOD Leu6, hSOD Ile6, hSOD Thr6, hSOD Ser6, hSOD Gln6, hSOD Asn6, hSOD Met6, hSOD SerIII, hSOD AlaIII, hSOD GlyIII, hSOD ValIII, hSOD LeuIII, hSOD IleIII, hSOD ThrIII, hSOD SerIII, hSOD GlnIII, hSOD AsnIII, hSOD MetIII, hSOD Gly6 GlyIII, hSOD Ala6 AlaIII, hSOD Leu6 LeuIII, hSOD Ile6 IleIII, hSOD Ser6 SerIII, hSOD Thr6 ThrIII, hSOD Gln6 GlnIII, hSOD Asn6 AsnIII, hSOD Met6 MetIII, hSOD Gly6 AlaIII, hSOD Gly6 ValIII, hSOD Gly6 LeuIII, hSOD Gly6 IleIII, hSOD Gly6 SerIII, hSOD Gly6 ThrIII, hSOD Gly6 GlnIII, hSOD Gly6 AsnIII, hSOD Gly6 MetIII, hSOD Ala6 GlyIII, hSOD Ala6 ValIII, hSOD Ala6 LeuIII, hSOD Ala6 IleIII, hSOD Ala6 SerIII, hSOD Ala6 ThrIII, hSOD Ala6 GlnIII, hSOD Ala6 AsnIII, hSOD Ala6 MetIII, hSOD Val6 GlyIII, hSOD Val6 AlaIII, hSOD Val6 LeuIII, hSOD Val6 IleIII, hSOD Val6 SerIII, hSOD Val6 ThrIII, hSOD Val6 GlnIII, hSOD Val6 AsnIII, hSOD Val6 MetIII, hSOD Leu6 GlyIII, hSOD Leu6 AlaIII, hSOD Leu6 ValIII, hSOD Leu6 IleIII, hSOD Leu6 SerIII, hSOD Leu6 ThrIII, hSOD Leu6 GlnIII, hSOD Leu6 AsnIII, hSOD Leu6 MetIII, hSOD Ile6 GlyIII, hSOD Ile6 AlaIII, hSOD Ile6 ValIII, hSOD Ile6 LeuIII, hSOD Ile6 SerIII, hSOD Ile6 ThrIII, hSOD Ile6 GlnIII, hSOD Ile6 MetIII, hSOD Ser6 GlyIII, hSOD Ser6 AlaIII, hSOD Ser6 ValIII, hSOD Ser6 LeuIII, hSOD Ser6 IleIII, hSOD Ser6 ThrIII, hSOD Ser6 GlnIII, hSOD Ser6 AsnIII, hSOD Ser6 MetIII, hSOD Thr6 GlyIII, hSOD Thr6 AlaIII, hSOD Thr6 LeuIII, hSOD Thr6 IleIII, hSOD Thr6 GlnIII, hSOD Thr6 AsnIII, hSOD Thr6 MetIII, hSOD Glu6 GlyIII, hSOD Glu6 AlaIII, hSOD Glu6 ValIII, hSOD Glu6 LeuIII, hSOD Glu6 SerIII, hSOD Glu6 ThrIII, hSOD Glu6 AsnIII, hSOD Glu6 MetIII, hSOD Asn6 GlyIII, hSOD Asn6 AlaIII, hSOD Asn6 ValIII, hSOD Asn6 SerIII, hSOD Asn6 ThrIII, hSOD Asn6 MetIII, hSOD Met6 GlyIII, hSOD Met6 AlaIII, hSOD Met6 ValIII, hSOD Met6 SerIII, hSOD Met6 ThrIII, hSOD Met6 GluIII, and hSOD Met6 AsnIII.

The N-terminus of the mutein may be acetylated (as in native hSOD) or lack acetylation depending upon the organism in which the mutein is produced. Bacterially produced mutein will lack such acetylation whereas mutein produced in yeast using the procedures described in EPA 84111416.8 are so acetylated. Muteins having such acetylation are preferred. Similarly, the mutein may be glycosylated or unglycosylated depending upon the organism and signaling sequence with which it is produced.

Genes encoding the muteins of the invention may be made via oligonucleotide synthesis and ligation, site directed mutagenesis of the DNA sequence shown in Figure 1 and/or by insertion of synthetic DNA fragments that encode the desired amino acid substitutions into a DNA sequence encoding native hSOD. Site directed mutagenesis techniques are well known in the art. See for instance, Smith and Gilliam In *Genetic Engineering Principles and Methods*, Plenum Press (1981) 3:1-32; Zoller and Smith, *Nucleic Acids Res* (1982) 10:6487-6500; and Brake et al, *Proc Natl Acad Sci USA* (1984) 81:4642-4646. The mutant genes may be inserted into suitable prokaryotic or eukaryotic replicons (a genetic element such as a plasmid, a chromosome, or a virus that behaves as an autonomous unit of polynucleotide replication within a cell), the resulting expression vectors incorporated into suitable host organisms or cells, the recombinant organism or cell grown under conditions that result in expression of the mutant gene, and the resulting mutein isolated from the host or, if secreted, from the growth medium using the same techniques as are described in EPA 84111416.8 to produce recombinant hSOD. The disclosure of that EPA is incorporated herein by reference.

In creating an expression vector, the mutant sequence is located in the vector with the appropriate control DNA sequences, which include a promoter, a ribosomal binding site, and transcriptional and translational stop codons. The positioning and orientation of the coding sequence with respect to the control sequences is such that the coding sequence is transcribed under the "control" of the control sequences: i.e., the promoter will control the transcription of the mRNA derived from the coding sequence, the ribosomes will bind at the ribosomal binding site to begin the translational process, and the stop codon used to terminate translation will be upstream from the transcriptional termination codon. In addition to control sequence, it may be desirable to

add regulatory sequences which allow for regulation of the expression of the mutant hSOD gene relative to the growth of the host cell.

The hSOD muteins of the invention may be used for the same purposes as hSOD. Because of their better thermostability, the muteins are more readily formulated with materials that require the use of elevated temperatures for blending or processing. The muteins may be used in human or veterinary medicine to treat (i.e. cure, alleviate or prevent) a variety of conditions. They are useful as antiinflammatory agents, chemopreventive agents to prevent oncogenesis and tumor promotion, protective agents to reduce cytotoxic and cardiotoxic effects of anticancer drugs or protect ischemic tissue. Like native hSOD, the muteins catalyze the reduction of superoxide radicals to hydroperoxide and molecular oxygen and may thus be used to reduce perfusion injury following ischemia, prolong the viability of excised isolated organ transplants, reduce injury on reperfusion following organ transplant or spinal cord ischemia, reduce cardiac infarct size, reduce spinal cord injury and treat bronchial pulmonary dysplasia.

For medical applications the mutein may be administered orally or parenterally to individuals in various dosage forms such as tablets, capsules, and injectables. When used to treat tissues *in vitro* the mutein will be added to the perfusion or culture medium. The mutein may be administered neat or admixed in effective amounts with pharmaceutically acceptable solids, semisolid or liquid vehicles such as albumins, globulins, dextran, Ficoll polymers, sugars, starches, and liposomes. Preferably the hSOD mutein is conveniently stored lyophilized with sugar, usually sucrose, usually in a ratio of 1:2 w/w. The lyophilized enzyme is conveniently reconstituted in a suitable diluent for the particular application. For example, to treat inflammatory joint disease the hSOD mutein may be reconstituted in physiologic saline in a volume convenient for intraarticular administration.

The dose of hSOD mutein administered to an individual will depend upon the nature of the individual being treated, the mode of treatment and the condition being treated. In general the amount administered must be sufficient to provide an enzymatically effective amount of the mutein at the desired site of treatment. In this regard when the mutein is administered systemically, larger doses will typically be required than when the mutein is administered locally at the site that requires treatment. By way of example, human patients having inflammatory joint disease are treated by a weekly intraarticular injection into a joint afflicted with the disease of a solution having hSOD mutein in a suitable diluent in an amount effective to reduce inflammation, usually 1 to 10 mg, more usually 2 to 6 mg. The injections are given weekly for a period of time sufficient to reduce inflammation, usually for 2 to 8 weeks, more usually for 4 to 6 weeks. Because the articular capsule limits leakage of the high molecular weight compound each afflicted joint should be treated with the required dosage. When used to minimize postischemic tissue damage the human patient is administered 10 mg to 1,000 mg, more usually 50 mg to 500 mg of hSOD mutein in a suitable diluent during the ischemic reaction. When the patient suffers ischemia due to a disease the solution is administered intravenously or intraarterially as a bolus dosage or a continuous infusion. In such situations the hSOD mutein may be administered in conjunction with fibrinolytic agents such as urokinase, streptokinase or tissue plasminogen activator (TPA). When ischemic damage is due to a surgical procedure, hSOD mutein is administered during surgery. This application finds particular use in organ transplant surgery where hSOD is preferably administered prior to reirrigation of the organ and is also useful in any other surgery where bloodflow to an organ is interrupted, such as open heart surgery.

The hSOD muteins may also be used in cosmetic compositions for skin or hair care as a protective agent for keratinic substances or to prevent oxidative degradation of components of such compositions. For instance they may be added to such formulations to maintain or improve skin or hair qualities such as softness, flexibility or elasticity or prevent oxidation of oxidizable or self-oxidizable substances such as dyes used in cosmetic preparations. The form of the mutein-containing cosmetic formulation may be solid (e.g. cleansing bars), semisolid (e.g. creams, gels, ointments) or liquid (e.g. sprays, lotions, shampoos). The amount of hSOD mutein in such formulations will normally be in the range of about 0.01% to 5% by weight, more usually 0.05% to 1% by weight. In addition to the hSOD mutein, the cosmetic preparation may contain: fatty carrier materials such as natural oils (e.g. olive oil, avocado oil, and the like), fatty acid esters such as stearin, glyceryl monostearate, ethyl palmitate, cetyl myristate, Isopropyl oleate and the like, alcohols such as cetyl alcohol or polyoxyethylenated fatty alcohols, waxes such as beeswax or synthetic waxes; dyes, perfumes, surfactants, preservatives, thickeners, or other additives conventionally included in cosmetics.

They may also be added to foods as preservatives to prevent oxidative degradation of food components. The following examples further illustrate the invention. These examples are not intended to limit the invention in any manner.

Construction of Yeast Plasmid pYSODA Encoding hSOD Ala6 Expression

The construction of plasmid pYSODA is depicted in Figure 2.

A DNA adaptor fragment (designated B in Figure 2) having the sequence shown below was synthesized.

NcoI 1 2 3 4 5 6 TaqI
 MetAlaThrLysAlaValAlaValLeuLysGlyAspGlyProValGlnGlyIleIleAsnPh
 CATGGCTACAAAGGCTGTTGCTGTTTTGAAGGGTGACGGGCCCGTTCAAGGTATTATTAACCTT
 CGATGTTTCCGACAACGACAAAACTTCCCACTGCCCCGGGCAAGTTCCATAATAATTGAAGC

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The fragment was phosphorylated on the coding (upper) strand only and was designed to encode the N-terminal sequence of hSOD with the codon for amino acid 6 altered to encode an alanine rather than a cysteine and have an NcoI site at its 5' end and a TaqI site at its 3' end.

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Plasmid pASIIr was linearized with NcoI and ligated to adaptor B. Plasmid pASIIr consists of the expression cassette of plasmid pASII (the hSOD gene fused to the N-terminus of the human proinsulin gene with a methionine codon at the junction of the two genes under the regulation of a hybrid inducible *S. cerevisiae* alcohol dehydrogenase 2-glyceraldehyde phosphate dehydrogenase (ADH2-GAP) promoter and the GAP terminator) in the S.R. 322 vector. Details of the composition and construction of plasmid pASII are described in commonly owned European Patent Application Serial No. 86104066.5, the disclosure of which as it relates to pASII is incorporated herein by reference. (The plasmid is designated pYASII in Serial No. 86104066.5.) The S.R. 322 vector is described in J Biol Chem (1985) 260:4384-4389. The ligation product was cut with Sall and the resulting large fragment was gel purified.

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Plasmid pSII/8 was cut with Sall and TaqI and a 660 bp TaqI - Sall fragment consisting of a portion of the hSOD gene fused to the N-terminus of the human proinsulin gene was gel isolated from the digest. Plasmid pSII/8 contains the hSOD gene fused to the N-terminus of the human proinsulin gene under the regulation of the GAP promoter and terminator. Details of its composition and construction are described in commonly owned European Patent Application Serial No. 86104066.5, the disclosure of which, as it relates to pSII/8, is incorporated herein by reference. (The plasmid is designated pYSII in Serial No. 86104066.5.)

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The 660 bp TaqI - Sall fragment was ligated to the linear pASIIr - adaptor B construct to produce plasmid pSI6. That plasmid was cut with NcoI and StuI and a 126 bp fragment gel isolated from the digest.

The plasmid pGAPSOD was cut with NcoI and StuI and vector DNA was gel purified. pGAPSOD is a yeast promoter vector containing the hSOD gene under the regulation of the GAP promoter and terminator. Details of its composition and construction are described in commonly owned European Patent Application Serial No. 84111416.8, the disclosure of which, as it relates to this plasmid, is incorporated herein by reference. (The plasmid is designated pPGAPSOD in Serial No. 84111416.8.) The 126 bp NcoI - StuI fragment from pSI6 was ligated with the vector DNA to form plasmid pSODA. pSODA was then cut with BamHI and a BamHI expression cassette having the hSOD gene with an Ala codon at amino acid 6 flanked by GAP promoter and terminator sequences was isolated. This expression cassette was then cloned in the expression orientation in plasmid pCI/I, which had been digested with BamHI and phosphatased, to produce pYSODA. Plasmid pCI/I is a derivative of pJDB219 (Beggs, Nature (1978) 275:104) in which the region corresponding to bacterial plasmid pMB9 in pJDB219 is replaced by pBR322.

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Construction of Yeast Plasmid pYSODS encoding hSOD SerIII Expression

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The mutagenesis of the hSOD CysIII codon to a ser-encoding triplet was accomplished using M13 site directed mutagenesis. The construction of the mutant M13 plasmid and related plasmids involved in the construction of pYSODS are depicted in Figures 3 and 4 and described below.

Plasmid pSODX8 (described in Nucleic Acids Res (1985) 13:2017-2034) was cut with StuI and Sall. An approximately 400 bp fragment was gel isolated and partially digested with Sau3A and a 327 bp hSOD fragment was recovered. Plasmid pSODX16 (described in Nucl Acids Res (1985) 13:2017-2033) was cut with StuI and BamHI and the large vector fragment was gel isolated from the digest. This vector fragment was ligated to the 327 bp hSOD fragment to produce plasmid pSODCFI.

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Plasmid pSI8, an hSOD-proinsulin fusion expression plasmid derived from pYASII (see above) and consisting of the expression cassette of pYASII in the S.R. 322 vector was cut with NcoI and StuI and a 124 bp fragment was gel isolated from the digest. That fragment was ligated with a 330 bp fragment isolated from pSODCFI digested with StuI and BamHI to yield a 454 bp NcoI - BamHI fragment. That fragment was amplified by cloning in plasmid pHBS6 (described in European Patent Application Serial No. 85105405.6, the disclosure of which, as it relates to pHBS6, is incorporated herein by reference).

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The phage vector M13p8 was cut with NcoI and BamHI and the resulting vector fragment ligated to the 454 bp hSOD fragment to produce the vector M13p8SOD-I. Single-stranded DNA was prepared from this vector to serve as a template for site directed *in vitro* mutagenesis using the following synthetic oligonucleotide primer 5'-GCCAATGATGGAATGGTCTCCTTGAGAGTGATATCACAGAATC-3'.

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The mutagenesis was carried out according to the procedure of Brake et al, supra. The mutant RF M13 clone containing the hSOD CysIII gene (designated M13p8SODCIIIS) was identified by screening with a ³²P-labeled probe having the sequence 5'-TTGAGAGTGATATCACAGAATC-3'.

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The mutant M13 clone M13p8SODCIIIS was cut with StuI and BamHI and a 330 bp StuI - BamHI fragment isolated from the digest. That fragment was ligated to a vector fragment prepared by digesting pSODCFI with StuI and BamHI. The resulting plasmid, pSODXCS, was digested with BamHI and a synthetic adaptor

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(designated (C) in Figure 5) having the sequence given below was ligated to the vector

BamHI

SalI

5 yileAlaGlnOC
GATCGCCCAATAAACATTCCCGGGGATGTAGTCTGAGGCCCTTAACATCATCTGTTATCCTGCTAG
CGGGTTATTTGTAAGGGCCCTACATCAGACTCCGGGAATTGAGTAGACAATAGGACGATCAGCT

10 The ligation product was then cut with StuI and a 260 bp StuI - SalI fragment was gel isolated. This fragment was then ligated to vector DNA prepared by digesting pGAPSOD with StuI and SalI to produce the plasmid pSODS. pSODS was then cut with BamHI and a fragment consisting of the hSOD CysIII gene flanked by GAP promoter and terminator sequences was isolated from the digest. That fragment was ligated into the vector pCI/I that had been cut with BamHI and phosphatased to produce the plasmid pYSODS.

15 Construction of Yeast Expression Plasmid pYSODAS for Expression of hSOD Ala6 SerIII

The construction of pYSODAS is diagrammed in Figure 6.

20 The above-described 260 bp fragment consisting of the BamHI - StuI fragment from pSODXCS with the adaptor (C) ligated to the BamHI end thereof was cloned into pSODA cut with StuI and SalI. The resulting plasmid pSODAS was cut with BamHI to produce a fragment consisting of the gene for hSOD Ala 6 SerIII flanked by GAP promoter and terminator sequences. That fragment was inserted into BamHI cut pCI/I to produce pYSODAS.

Preparation of Recombinant Yeast Strains

25 Plasmids pYSODA, pYSODS, and pYSODAS were transformed into yeast strain 2150-2-3 as described in PNAS USA (1978) 75:1929-1933 to produce recombinant strains that produce hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII.

Yeast Growth and hSOD Mutein Purification

30 Ten liter cultures of yeast strains producing hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII were grown by inoculating 10 L of YEPD medium containing 3 mM CuSO₄ with 500 ml of starter culture in minimal glucose medium lacking leucine (Sherman, F., Fink, G.R., & Hicks, J.B., Methods in Yeast Genetics, (1982), Cold Spring Harbor, New York) supplemented with 50 µM CuSO₄ and grown to OD₆₅₀ ~ 20.

35 Yeast pastes (100-250 g wet weight) were lysed in 20 mM Tris-HCl, pH 8 (buffer), using a Dymomill Bead Disrupter. The lysate was centrifuged, and the pellet washed twice with buffer. The washes were pooled with the original supernatant. After centrifugation, approximately 1 liter of extract was obtained, adjusted to pH 8 with NaOH, and stored at -20°C. The extract was thawed and heated for 2 hr at 65°C. After centrifugation to remove the precipitated protein and debris, 850 ml of clarified extract was obtained. Buffer was added to final volume of approximately 8.5 liters, resulting in a conductivity of 1.2 mmho. This was loaded onto a 400 ml column of DEAE-Sepharose. The column was washed with the same buffer, and eluted with a gradient up to 0.15 M NaCl in buffer. The peak was collected in 3 pools: A, which contains the bulk of the material from the center of the peak; B, containing the early side fractions; and C, containing the later side fractions. The pools were sterile filtered and aliquots stored at 4°C and at -20°C. Photographs of the gels of these fractions are shown in Figure 7.

45 The procedure resulted in a 2.5-fold purification, yielding protein at least 95% pure (see figure 7). Due to the high expression level (approximately 40% of the total protein), 2.5-fold purification is all that is possible. The overall yield is ~ 60%; the heating step resulted in an 83% yield, and the column ~ 70%. The heating step is valuable in that it purifies the hSOD mutein, and also clarifies the extract which otherwise is turbid with debris that is difficult to remove. The DEAE-Sepharose column removes the remaining contaminating proteins and other nonproteinaceous contaminants such as lipid, carbohydrate, and nucleic acid.

50 The purified hSOD mutein was compared to that present in the crude extract to ensure that not only was the activity present, but that the protein itself was unmodified. This is particularly important because of reactions that could occur during the heat step. By native gel electrophoresis in agarose gels, it is usually possible to separate 5 charged isomers to hSOD. These are called forms +2, +1, 0, -1 and -2. In fact, we found by native polyacrylamide gel and agarose gel electrophoresis, the hSOD Ala6 SerIII is apparently unaltered after heating. As seen in Figure 8A, when extract is heated for 2 hr at 65 or 70°C, the pattern of bands seen after agarose gel electrophoresis is only slightly changed. This is a loss of some minor, very rapidly migrating species, but otherwise there is no change.

55 The specific activity of hSOD Ala6 SerIII (3400 U/mg) is approximately the same as wild type hSOD (3500 U/mg) as assayed by the pyrogallol method. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur J Biochem (1974) 47:469-474).

Thermal Stability of hSOD, hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII

60 Analysis of the thermostability of the hSOD muteins made in yeast was performed by heating crude extracts at 80°C for 10 min and 25 min and comparing the superoxide dismutase activity of the heated SODs with that of the unheated control on an SOD activity gel as shown in Figure 9 (Beauchamp, C. and Fridovich, I., Analyt

Biochem (1971) 44:276-287). The results depicted in Figure 9 indicate that hSOD Ala6 SerIII is detectably more thermostable than hSOD and hSOD Ala6 and hSOD SerIII. hSOD Ala6 and hSOD SerIII also appear to be more thermostable than wild type hSOD.

Thermal Stability of hSOD Ala6 SerIII

A. At pH 4.0. The thermal stabilities of purified recombinant hSOD, bovine SOD and hSOD Ala6 SerIII were determined in 20 mM sodium acetate, pH 4, at a protein concentration of 20 µg/ml. After heating, the SOD activity was measured by the pyrogallol method. Figure 10 shows the melting curve seen after 1 hr of heating at temperatures from 50° to 70°C. Note that hSOD Ala6 SerIII exhibits the most resistance to thermal denaturation under these conditions, followed by bovine SOD, and then by hSOD. After 1 hr at 60°C, hSOD Ala6 SerIII has retained 86% of its activity, while the bovine SOD and hSOD have retained 68% and 48% activity, respectively.

Figure 11 shows the rate of denaturation at 60°C. Here the differences after 1 hr are less than the previous experiment (69% vs. 62% vs. 56%), but the relative stabilities are consistent with the earlier result.

The increased stability of the hSOD Ala6 SerIII is especially evident after heating at 70°C (data not shown). Here, after 30 min, the hSOD Ala6 SerIII retained 29% of its activity vs. ~ 10% for the other SODs. And even after 90 min at 70°C, the hSOD Ala6 SerIII still had 25% of its original activity while the others had less than 10% activity.

B. At pH 7.8. The experimental conditions were identical to those at pH 4, except that the buffer was 0.1 M sodium phosphate, pH 7.8. Figure 12 shows the rates of inactivation at 70°C. Here, the differences in stability are dramatic; the hSOD Ala6 SerIII is most stable, followed by bovine SOD and then hSOD. These are the same relative stabilities seen at pH 4, but at pH 7.8 the hSOD Ala6 SerIII now has a 10-fold longer half-life at 70°C than hSOD (175 vs. 16 min). It is also more than 2-fold more stable than bovine SOD (175 vs. 84 min).

Modifications of the above described modes for carrying out the invention that are obvious to those of ordinary skill in the fields of protein chemistry, genetic engineering, medicine and related fields are intended to be within the scope of the following claims.

Claims

1. A mutein of human Cu/Zn superoxide dismutase in which at least one of the cysteine residues at positions 6 and III is replaced with an uncharged amino acid.
2. The mutein of claim 1 wherein said uncharged amino acid is acyclic.
3. The mutein of claim 2 wherein the side chain of the uncharged acyclic amino acid is hydrogen, an aliphatic side chain or a hydroxylic side chain.
4. The mutein of claim 1, 2 or 3 wherein the N-terminus of the mutein is acetylated.
5. The mutein of claim 1, 2, 3, or 4 wherein only one of said cysteine residues is replaced with an uncharged amino acid.
6. The mutein of claim 5 wherein the one of said cysteine residues is at position 6.
7. The mutein of claim 5 wherein the one of said cysteine residues is at position III.
8. The mutein of claim 1, 4, 5, 6, or 7 wherein the uncharged amino acid is glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, or methionine.
9. The mutein of claim 1, 2, 3, or 4 wherein both of the cysteine residues are replaced with an uncharged amino acid.
10. The mutein of claim 9 wherein the uncharged amino acid is glycine, valine, alanine, leucine, isoleucine, serine, threonine, asparagine, glutamine, or methionine.
11. The mutein of claim 9 wherein the cysteine residue at position 6 is replaced with alanine and the cysteine residue at position III is replaced with serine.
12. A method of enhancing the thermostability of human Cu/Zn superoxide dismutase comprising replacing at least one of the cysteine residues at positions 6 and III with an uncharged amino acid.
13. The method of claim 12 wherein only the cysteine residue at position 6 is replaced and the uncharged amino acid is serine.
14. The method of claim 12 wherein only the cysteine residue at position III is replaced and the uncharged amino acid is serine.
15. The method of claim 12 wherein the cysteine residue at position 6 is replaced with alanine and the cysteine residue at position III is replaced with serine.
16. A pharmaceutical composition comprising an enzymatically effective amount of the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 admixed with a pharmaceutically acceptable carrier.
17. A cosmetic composition comprising an enzymatically effective amount of the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 admixed with a carrier.
18. DNA encoding the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.
19. An expression vector for expressing the DNA of claim 18 in an organism comprising the DNA of claim 18 operably connected to DNA that enables the expression of the DNA of claim 18 in the organism.
20. A host organism containing the vector of claim 19 which permits the expression of said mutein-encoding DNA.

			1									10		
GAGTT	Met	Ala	Thr	Lys	Ala	Val	Cys	Val	Leu	Lys	Gly	Asp	Gly	
	ATG	GCG	ACG	AAG	GCC	GTG	TGC	GTG	CTG	AAG	GGC	GAC	GGC	
						20								
Pro	Val	Gln	Gly	Ile	Ile	Asn	Phe	Glu	Gln	Lys	Glu	Ser	Asn	Gly
CCA	GTG	CAG	GGC	ATC	ATC	AAT	TTC	GAG	CAG	AAG	GAA	AGT	AAT	GGA
		30										40		
Pro	Val	Lys	Val	Trp	Gly	Ser	Ile	Lys	Gly	Leu	Thr	Glu	Gly	Leu
CCA	GTG	AAG	GTG	TGG	GGA	AGC	ATT	AAA	GGA	CTG	ACT	GAA	GGC	CTG
						50								
His	Gly	Phe	His	Val	His	Glu	Phe	Gly	Asp	Asn	Thr	Ala	Gly	Cys
CAT	GGA	TTC	CAT	GTT	CAT	GAG	TTT	GGA	GAT	AAT	ACA	GCA	GGC	TGT
		60										70		
Thr	Ser	Ala	Gly	Pro	His	Phe	Asn	Pro	Leu	Ser	Arg	Lys	His	Gly
ACC	AGT	GCA	GGT	CCT	CAC	TTT	AAT	CCT	CTA	TCC	AGA	AAA	CAC	GGT
						80								
Gly	Pro	Lys	Asp	Glu	Glu	Arg	His	Val	Gly	Asp	Leu	Gly	Asn	Val
GGG	CCA	AAG	GAT	GAA	GAG	AGG	CAT	GTT	GGA	GAC	TTG	GGC	AAT	GTG
		90										100		
Thr	Ala	Asp	Lys	Asp	Gly	Val	Ala	Asp	Val	Ser	Ile	Glu	Asp	Ser
ACT	GCT	GAC	AAA	GAT	GGT	GTG	GCC	GAT	GTG	TCT	ATT	GAA	GAT	TCT
						110								
Val	Ile	Ser	Leu	Ser	Gly	Asp	His	Cys	Ile	Ile	Gly	Arg	Thr	Leu
GTG	ATC	TCA	CTC	TCA	GGA	GAC	CAT	TGC	ATC	ATT	GGC	CGC	ACA	CTG
		120										130		
Val	Val	His	Glu	Lys	Ala	Asp	Asp	Leu	Gly	Lys	Gly	Gly	Asn	Glu
GTG	GTC	CAT	GAA	AAA	GCA	GAT	GAC	TTG	GGC	AAA	GGT	GGA	AAT	GAA
						140								
Glu	Ser	Thr	Lys	Thr	Gly	Asn	Ala	Gly	Ser	Arg	Leu	Ala	Cys	Gly
GAA	AGT	ACA	AAG	ACA	GGA	AAC	GCT	GGA	AGT	CGT	TTG	GCT	TGT	GGT
		150				153								
Val	Ile	Gly	Ile	Ala	Gln	OC								
GTA	ATT	GCG	ATC	GCC	CAA	TAA								

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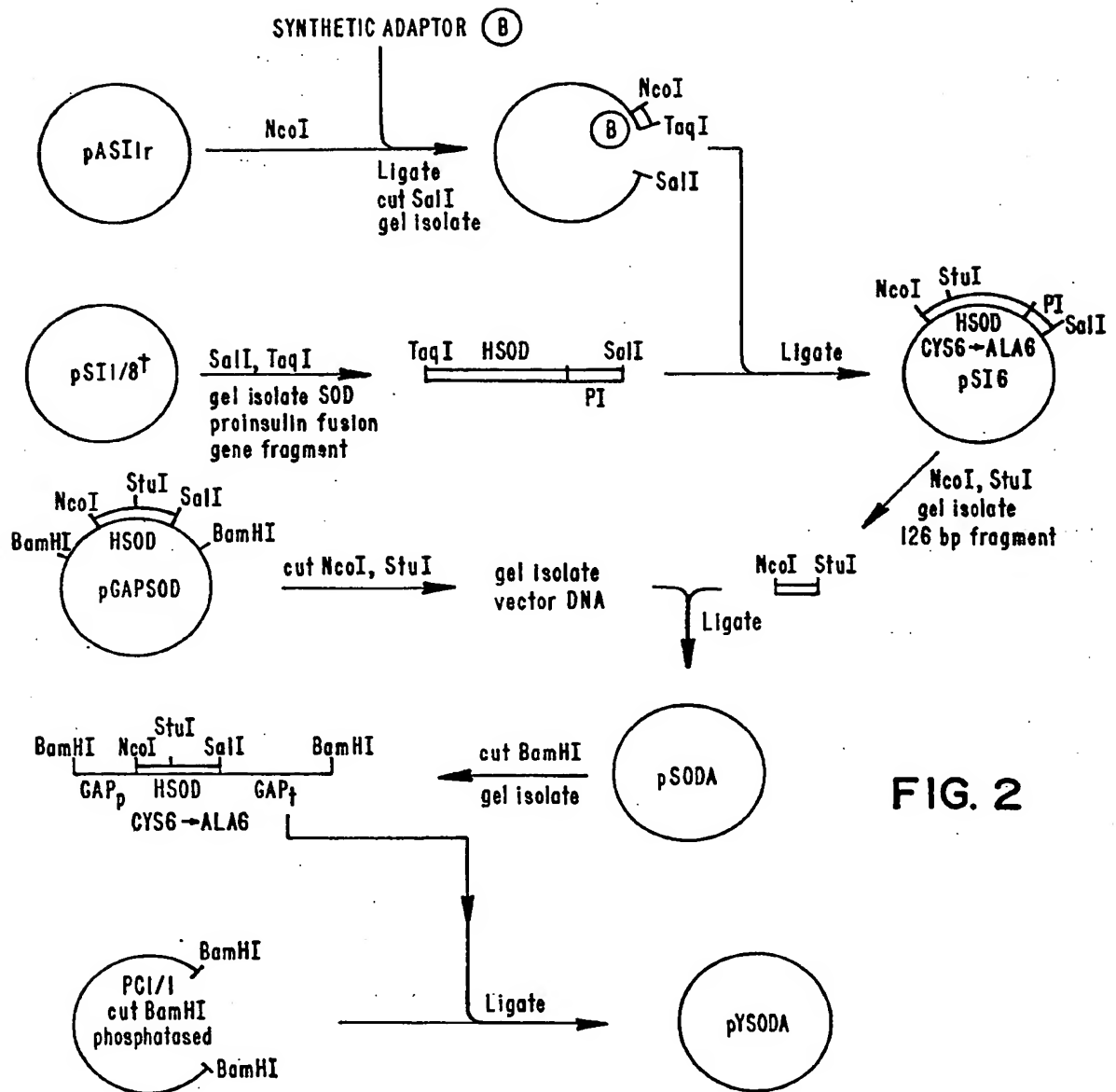


FIG. 2

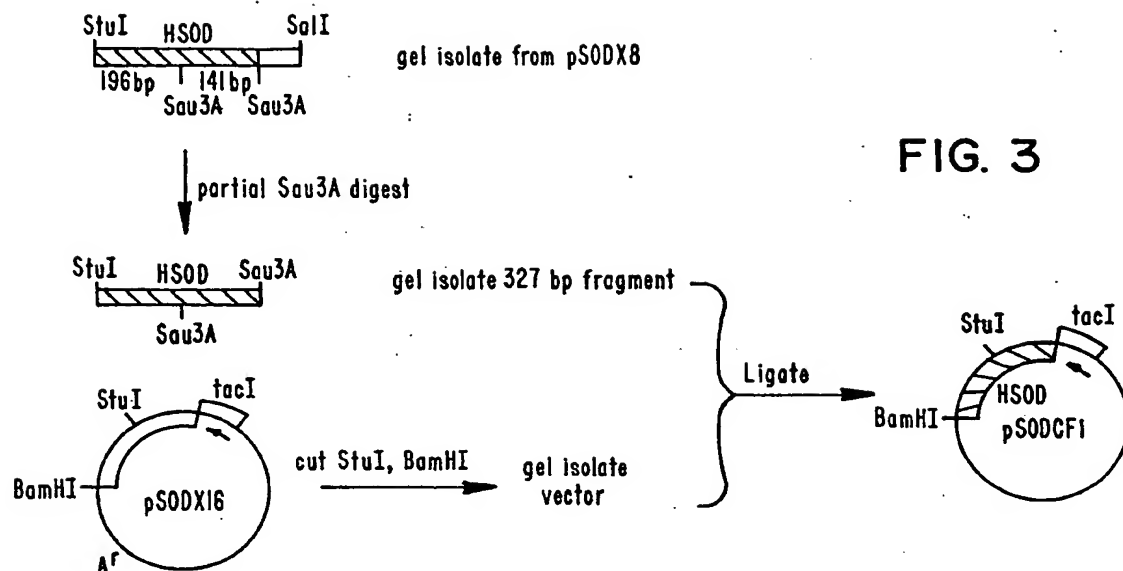


FIG. 3

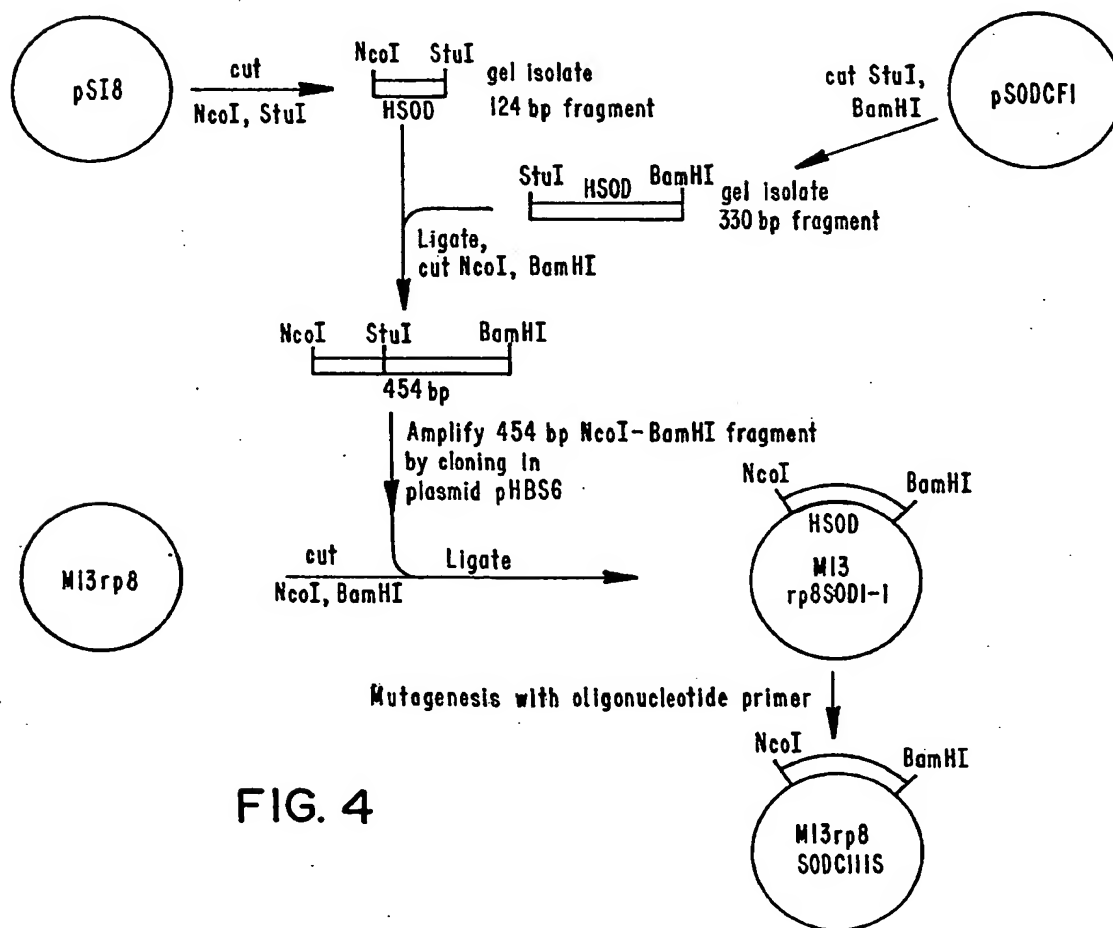
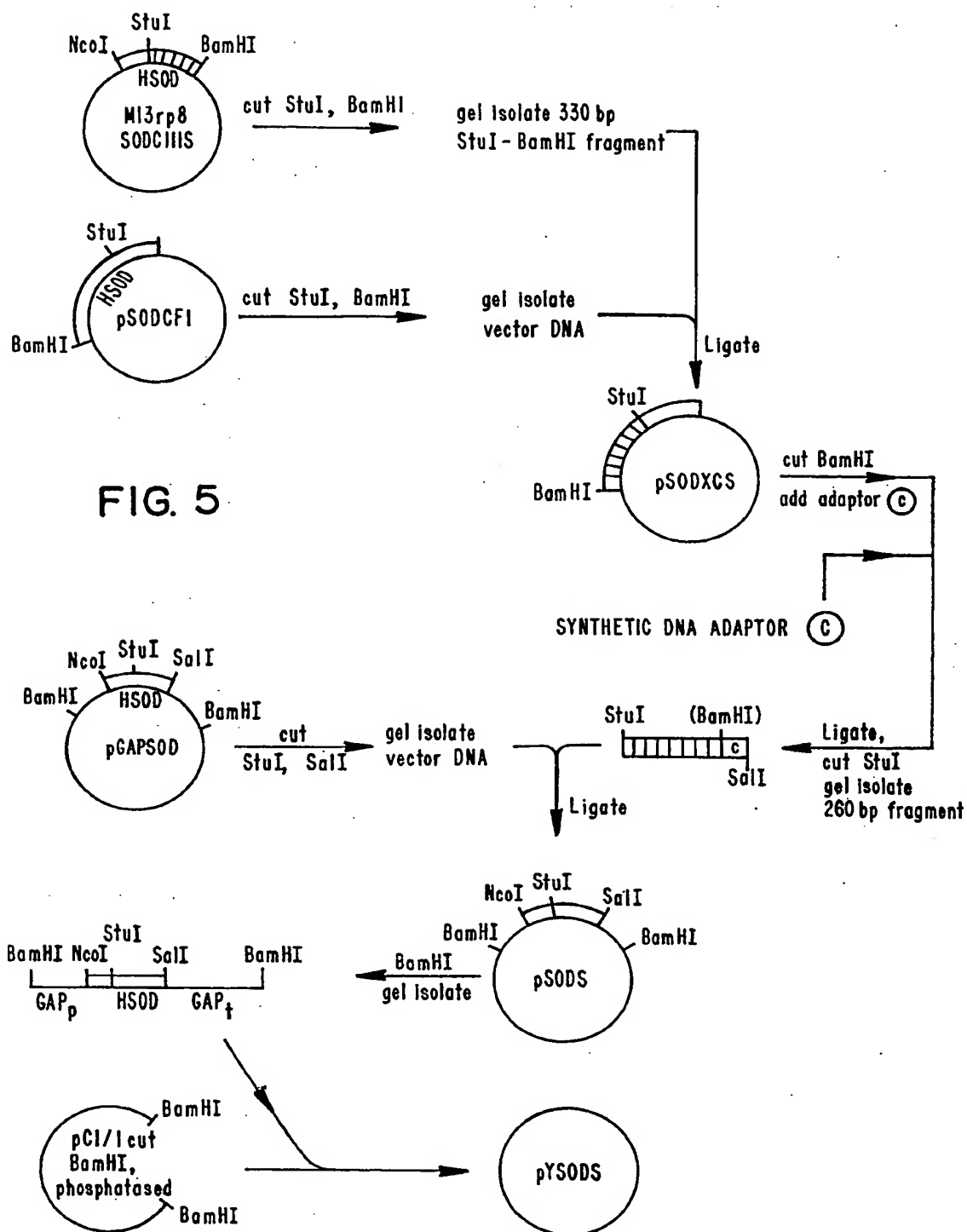


FIG. 4

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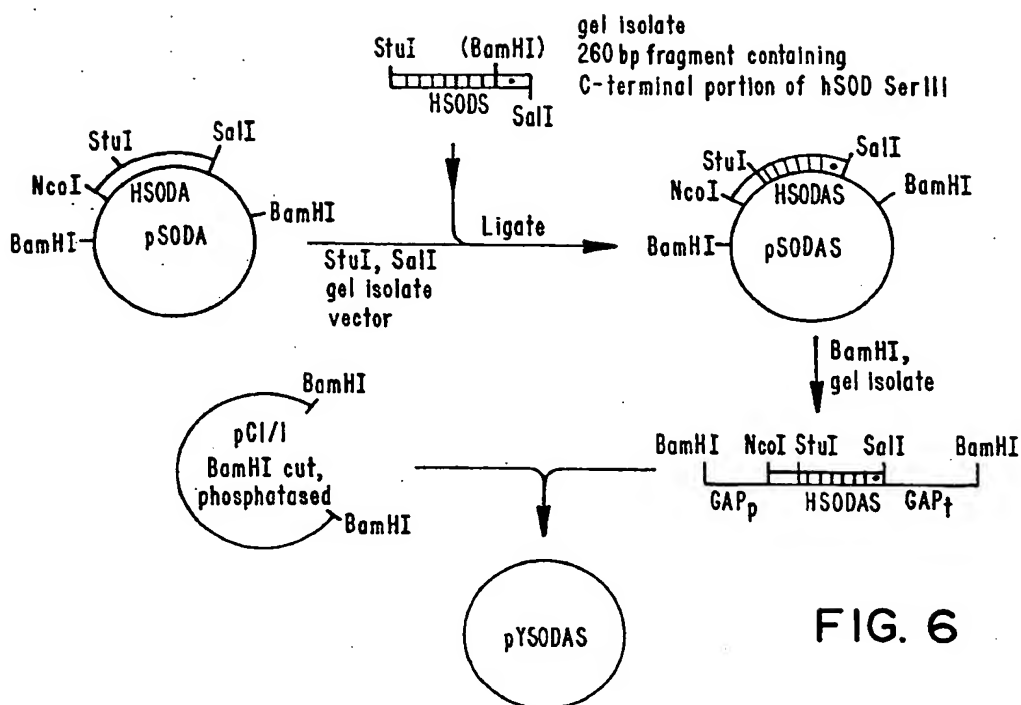
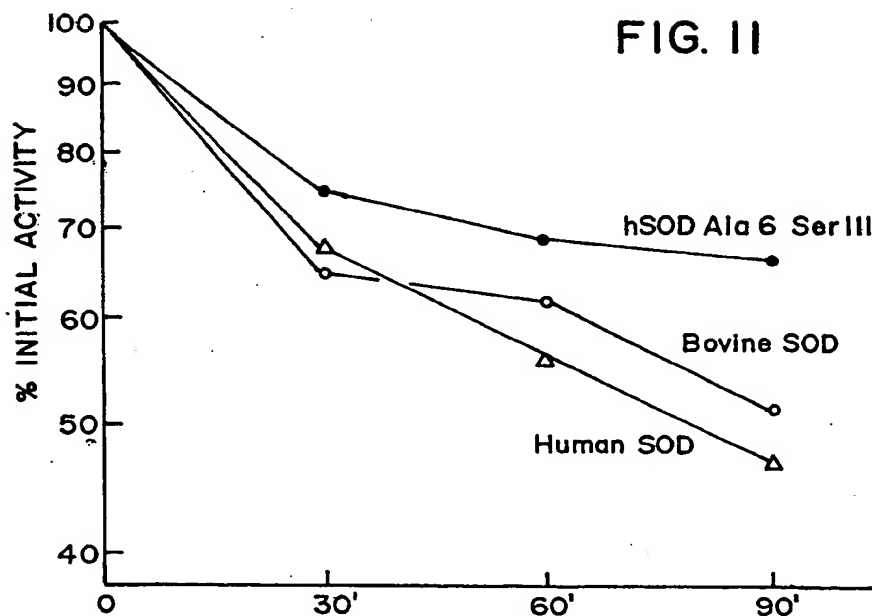


FIG. 6



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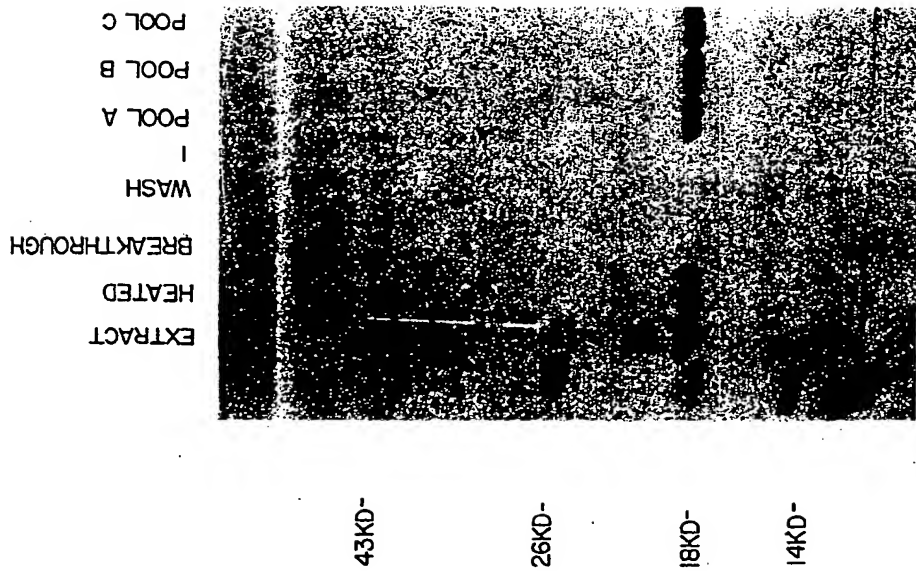


FIG. 7

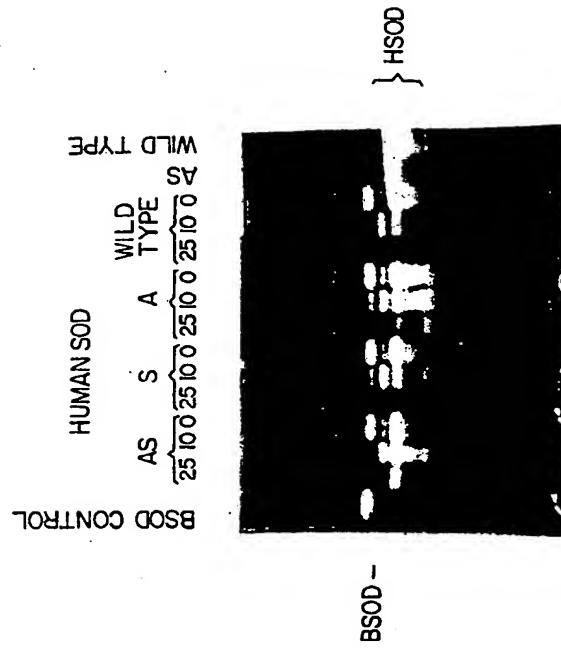


FIG. 9

08 07

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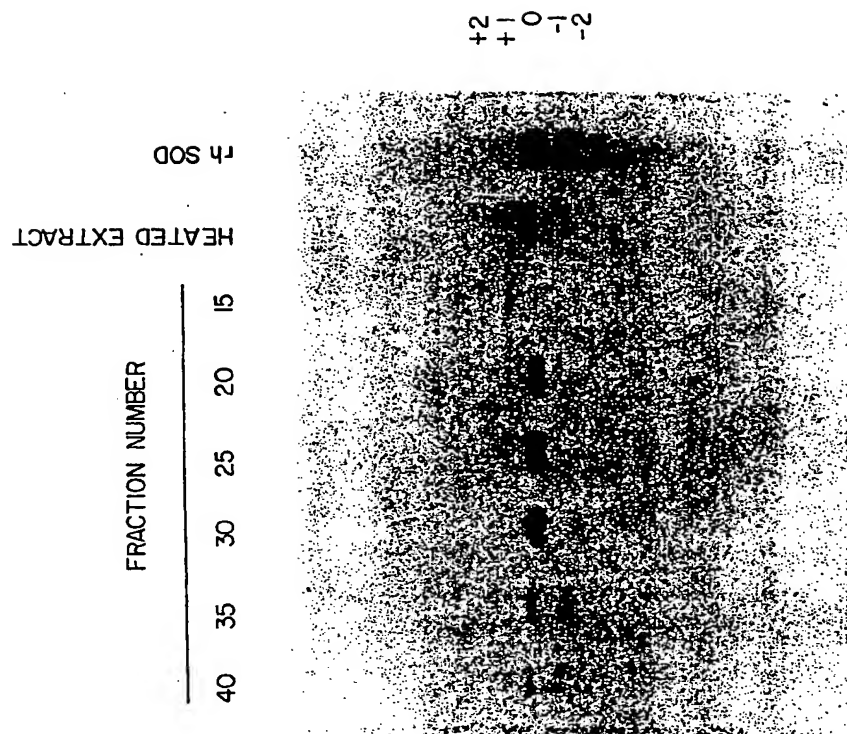


FIG. 8B

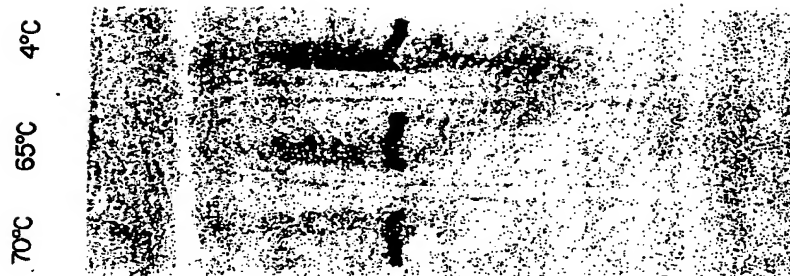


FIG. 8A

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